

Manuscript nanomaterials-875356

Point-by-point response to reviewers

Dear Editor and Reviewers,

Our manuscript entitled "In vitro impact preliminary assessment of airborne particulate from metalworking and woodworking industries" has been corrected according to expert's all comments, objections, comments and suggestions.

Reviewer 1:

The study reports on the effects of airborne material collected from metalworking and woodworking industries on 3D airway epithelial tissues with regard to cell toxicity, mRNA levels for Casp-3, gelsolin and IL-6 and H & E staining. Cell viability was not significantly affected but mRNA levels for gelsolin and IL-6 were reduced. The studies are technically sound and well designed.

Comments:

1. Use of 3D airway tissues in air-liquid culture system is a strength of the study as it may closely mimic in vivo conditions. Do the tissues contain macrophages? Please specify the sex of the donor. Do you know if the particles are taken up and accumulated by tissues?

Thank you for the question. No, tissues does not contain macrophages. Tissues are commercially available from MatTek Corp., and product specifications does not include information about donor age and sex. Additional experiments are needed (e.g. microscopy with labelled particles, electronmicroscopy) to describe in detail interaction of particles with cells and tissues. The information about commercially available product were specified in the article.

2. Do you know if the collected particulates contain materials other than wood or metal particles? These particles may modify the effects of wood and metal particles. Please comment.

Thank you for the comment.

Content of particulate matter was analysed and was published in the following articles, also mentioned in current articles:

- I.Pavlovska, Ž.Martinsonē, A.Ramata-Stunda, I.Vanadzins, I.Martinsonē, A. Seile. Comparison of biological markers in aerosol-weighed workplaces. *Journal of Nanoparticle Research* 21(7), Article No. 138, pp.1-10, 2019.
- N.Kurjane, T.Zvagule, I.Martinsonē, I.Vanadzins, I.Pavlovska. The Effect of Different Workplace Nanoparticles on the Immune Systems of Employees". *Journal of Nanoparticle Research*. Vol 19 (9) 320, 2017.
- Pavlovska, I., Martinsonē, Z., Vanadzins, I., Martinsonē, I., Seile, A., Sudmalis, P. Occupational exposure parameters for characterization of nanoparticulate matter toxicity: Metal versus wood processing. *Process Safety and Environmental Protection* 102, 2016.

During these specific occupational processes (metal- and woodworking), there was not formation of additional unknown chemical compound regarding our study results.

3. 2 – can you provide statistical significance?

No statistically significant changes were observed for tissue viability (n=3). Gene expression analyses did not have biological replicates due to limited availability of tissues (n=1). Please see the text. It was corrected.

4. 3 – Provide n, number of independent experiments performed and statistical significance.

N=3 (technical) for viability, n=1 for gene expression. Please see the text. It was corrected.

5. Provide data on protein levels for caspase-3, gelsolin and IL-6? Protein data would help understand the tissue responses better.

Thank you very much for your suggestion, we took note this fact.

Analysis of protein levels were not performed in this study and could be provided during next research steps.

6. 4 – is this from a single experiment? Did you do replicate experiments? There appears to be significant changes in tissue appearance, yet it is concluded otherwise. C-8h, C-72 h and M- 8 h and M-72 h tissues have reduced thickness with reduced number of cells, whereas W-4 h tissues have enlarged cells and W-8h tissues appear more like C-4h tissues. Please comment on these changes.

Thank you for your comment.

It is a single experiment. Differences seen in the microscopy pictures are due to different settings for taking pictures. Scale has been adjusted for pictures to be more comparable. Please see the text.

7. It is concluded that the particles have negative effects on cell metabolism. It would be more appropriate to write that the particles have negative effects on cell viability. Can be changes this way.

It can be changed that particles have negative effect on viability, however it should be taken in to account that MTT assay evaluates metabolic activity of cells and tissues. Cellular oxidoreductases reduce MTT to form formazan dye, so MTT assay reflects metabolic activity as well. Please see the text, all corrections were done.

8. The text needs editing for minor spelling errors.

The text was reviewed for spelling errors by authors.

9. Line 115 - 0.674 mg should be changed to 0.674 mg (microgram)?

Here are milligrams in text.

10. 116 – Change 25 ml to 25 µl (microliter).

25 µl was changed to 25 µL in Line 116, Line 122, and in the Figure 1.

11. Line 185 – Change to “Results and Discussion”

The “Results and Discussions” was changed to “Results and Discussion”. Thank you for your remark.

12. Line 246 – change to “caspase-3 gene coding for protease”

Thank you for suggestions. It was corrected, please see the text.

13. Line 247 – change to “pollutants from”

Thank you for suggestions. It was corrected, please see the text.

14. Line 260 – Change to “pleiotropic”

It was corrected, please see the text. Thank you for your remark.

15. Line 299 – provide reference for your previous studies

The references of previous studies are already included in the Reference List.

- 1.I.Pavlovska, Z.Martinsone, I.Vanadzins, I.Martinsone, A.Seile, P.Sudmalis. Occupational exposure parameters for characterization of nanoparticulate matter toxicity: Metal versus wood processing. *Process Safety and Environmental Protection*, pp. 230-237, 2016.
- 2.I.Pavlovska, Ž.Martinsone, A.Ramata-Stunda, I.Vanadzins, I.Martinsone, A.Seile Comparison of biological markers in aerosol-weighed workplaces. *J. Nanopart. Res.* 21(138), 2019.

16. Change hematosilin to hematoxylin

There are hematoxylin in the text already. Thank you for your remark.

All your critical remarks have been taken into account, thanks for them. Now, in our opinion they have been corrected. Please see the text.

Reviewer 2:

1. To investigate the occupational hazard represented by airborne particulate matter in metalworking and woodworking industries, the authors collected particles from both sites by Midget Impingers and exposed a 3D epithelial airway cell model to these particles. Readout parameters from the cell model are presented as cellular viability by MTT assay and RT-qPCR analysis of gelsolin, caspase-3 and IL-6 as well as tissue morphology by hematoxylin/eosin staining. The expression of gelsolin was found to be reduced in both occupational settings and IL-6 RNA was reduced by metalworking related particles. The article generally takes up a very central occupational health topic in is certainly interesting in terms of results as well as used methodology.

2. Spelling and grammar of the article are good, there are only minor corrections required in the text.

The text was reviewed for spelling errors by authors.

3. Data presentations in figures need improvement to reach an international standard level. First, if there are error-bars in columns, the authors should use a y-axis scale that allows for clearly showing the error-bars.

All pictures in the article were corrected. Thank you for your suggestion.

4. Second, the author need to indicate in the figure and in the text, if the error-bar represents SD or SEM.

Thank you for the comment. For the viability, it is SD from technical replicates.

5. Third, the authors need to indicate N (number of independent experiments) and n (number of repeats per independent experiment in figures and text. For in-vitro experiments, an N = 1 is not acceptable with very few exceptions.

Viability n=3 from technical replicates, gene expression n=1. Thank you very much for your suggestion, we took note this fact.

6. Next, the pattern of the columns is confusing, as one of the patterns in the legend does not match any column and one is obviously matching the wrong column.

Thank you for the comment. It was corrected, please see the text.

7. In Fig.2, the line indicating 100 % is not on mark with the y-axis.

Thank you for the comment. It was corrected, please see the text.

8. All visible differences should be tested for statistical significance and significance or absence of significance needs to be indicated in the figures.

No statistically significant changes were observed for tissue viability (n=3). Gene expression analyses did not have biological replicates due to limited availability of tissues (n=1). Please see the text. It was corrected.

9. Histological images (Fig. 4) are missing scale bars and the cell nuclei are indicating that the size representation is not uniform for all nine images shown. This makes comparisons between images nearly impossible.

Now it is Figure 5. Thank you for the comment, all corrections were made. Please see the text. It was corrected.

10. The labels for the x-axis in Fig. 2 are misleading, because the authors did not test for exposure times but particle concentrations. This is a result of the concept the authors choose to analyze different times of exposure. This concept is, at least, questionable, as it represents exposure time in a highly simplified form of particle concentrations and does not consider the time-dependence of the cellular system in the response to this exposure. Lung deposition model calculations are therefore complicated as they need to consider extrusion processes going on during exposure as well as the effect of mucus and differences in higher and lower lung regions. That this simplification does not work out for the given model can be seen in Fig.2 for woodworking particles: The columns show, that the viability was going down between 4h and 8h of exposure and then back to normal at 72h. This would suggest a recovery of metabolic cell viability between 8h and 72h, because to get to 72h, cells need to survive 8h first. However, this is not the case, as only different concentrations of particles were tested and cells did not experience the condition at 8h before 72h.

Labels on the x axis correspond to the experimental design shown in Figure 1.

11. Line 80: "...processes were used many to assess..."

The Line 80 was corrected: "...processes were used to assess organic particles."

12. Before this study can be recommended for publication, the authors need to address these conceptual problems and remove all forms of misleading presentations (i.e. clearly indicate that different concentrations of particles were tested, representing occupational exposures). In addition, statistical analysis is required for in vitro experiments and the quality of graphical data presentation should reach international standards.

All your critical remarks have been taken into account, thanks for them. Now, in our opinion they have been corrected. Please see the text.